

## [RAPID COMMUNICATION]

**Strictly Thymus-dependent Tolerance Induction in Immunologically Competent *Xenopus* Larvae**

SHIN TOCHINAI

*Division of Biological Sciences, Graduate School of Science,  
Hokkaido University, Sapporo 060, Japan*

**ABSTRACT**—Larvae of J strain South African clawed frog *Xenopus laevis* (MHC haplotype; JJ) can easily be rendered tolerant, during metamorphosis, of semi-xenogeneic adult skin grafts from interspecific hybrid (JB) made between JJ and *X. borealis* (BB). If larvae were thymectomized several days before, at the same time as, or within 1 week after the skin grafting, tolerance was no longer induced. Tolerance induction was restored by grafting late larval or adult thymuses before or within 1 week after skin grafting. Early larval thymuses were ineffective for this restoration. Overall results indicate that the tolerance induced during metamorphosis is ascribable to a suppression exerted on a competent immune system in the presence of differentiated thymus.

**INTRODUCTION**

The clonal deletion resulting in the tolerance of transplantation antigens expressed in the thymus has been well established as a major mechanism [13]. On the other hand, the tolerance of extrathymic antigens has not thoroughly been understood, while in several cases it have been shown to be mediated either by the clonal deletion or anergy [6]. Considering the limitations to account for many aspects of tolerance, however, it will clearly be important to speculate the third mechanism, i.e., suppression. Although the suppressor cell mediated regulation is strongly questioned these days, the absence of the suppression has not been concluded either [3]. In this connection, a very interesting phenomenon has been reported quite recently that the suppressive activity was exerted

by CD4<sup>+</sup> T cells that should generally be classified as helper [12]. Under this condition, any efforts will be justified where the mechanisms of immunological suppression can be experimentally analyzed.

During metamorphosis, the immunocompetent amphibian larvae must recognize the newly appearing adult-type antigens as self and consequently tolerate them. Here, I will present a new model system where transplantation tolerance of semixenogeneic adult skin is induced in immunocompetent tadpoles of *Xenopus laevis* during metamorphosis.

**MATERIALS AND METHODS**

The animals used in the present study were from J strain of the South African clawed frog *Xenopus laevis*, designated JJ to reflect its homozygosity at the major histocompatibility complex (MHC) loci, and from partially inbred colony of *Xenopus borealis* (BB). Artificial inseminations were made to produce normal JJ embryos or interspecific hybrid (JB) between *X. laevis* females and *X. borealis* males. Developmental stages of embryos and larvae were determined according to the Normal Table of Nieuwkoop and Faber [9]. Animals were maintained at 23±1°C throughout the experiments.

To thymectomize early larvae at stages 44-46 (4-5 days after fertilization), thymic rudiment of the animal was cauterized with a sharpened needle connected to high frequency electrosurgical apparatus [15]. Thymectomy at later larval stages was

done similarly using a blunt needle.

Ventral skin grafts from BB or JB adults were transplanted either on adult or larval JJ animals according to the technique described previously [10]. Grafting of the thymus or the spleen was accomplished by simply inserting the organ subcutaneously in larvae and adults.

Health of skin grafts was observed frequently under a stereomicroscope. Rejection was monitored by the destruction of guanophores (white pigment cells) in the grafted ventral skin [8, 10]. Scoring was made 1 month after metamorphosis or 2 months after organ (or skin) grafting, when the fate of skin grafts can be classified into either rejection (completely destroyed) or tolerance (healthy at least partially).

## RESULTS AND DISCUSSION

In JJ adults, BB and JB skin grafts were rejected in about 20 days at 23°C, a comparable time to that in allograft rejection among *X. laevis*. Early larval thymectomy, at stages 44–46 before the thymus differentiation occurs, completely abrogated the development of BB and JB graft rejecting capacity as shown previously [4]. This indicates that the rejection of (semi)xenogeneic skin grafts is dependent on the T cell dependent responses like allograft rejection [5].

Unlike in adult frogs, skin grafts from JB adults were usually tolerated by stage 54–56 JJ tadpoles beyond metamorphosis, if the size of the graft was large enough (more than 4 mm<sup>2</sup>). Most of the smaller grafts (1 mm<sup>2</sup>) were eventually rejected within 1 month after metamorphosis, showing that the metamorphosing animals were competent to destroy JB skin. On the other hand, adult BB skin grafts were never tolerated even when grafts larger than 4 mm<sup>2</sup> were used. The situation concerning the size of grafts and MHC disparity between hosts and donors is quite comparable with that observed in allogeneic combinations [1, 8].

The most intriguing finding in the present study is the requirement of the thymus during the tolerance induction. Host larvae were thymectomized at various days before or after the JB skin grafting. The thymus at these stages has reportedly ceased to function since thymectomy is no

longer effective to abrogate the capacity for skin allograft rejection [5]. Nevertheless, if larvae were thymectomized several days before, at the same time as, or within a week after the skin grafting, tolerance was no longer induced (Table 1). Tolerance induction was restored by grafting stage-matched JJ thymuses to these tadpoles before or within several days after the skin grafting (Table 2). For this restoration, not only the thymuses from stage-matched larvae but also those from adult frogs were effective. On the other hand, less

TABLE 1. Requirement of the thymus for tolerance induction

days of thymectomy done before (–) or after (+) skin grafting	number of animals made tolerant
–14	0/10
–1	0/10
0	0/10
+7	1/10
+14	10/10
+21	10/10
+42	10/10

JJ larvae were thymectomized before (–), after (+), or at the same time (0) as JB adult skin grafting on stage 54–56 tadpoles. The rates of tolerance induction were scored one month after metamorphosis.

TABLE 2. Tolerance induction after thymus grafting

days of thymus grafting made after skin grafting in thymectomized tadpoles	number (%) of animals made tolerant
—	0/10 ( 0)
0	10/10 (100)
2	6/ 7 ( 86)
5	4/16 ( 25)
9	3/ 7 ( 43)
21	0/10 ( 0)
0 (adult thymus)	10/10 (100)
0 (stage 48 thymus)	0/10 ( 0)

JJ larvae had been thymectomized 14 days before the skin grafting, and were grafted with a pair of JJ thymuses from stage-matched larvae unless otherwise stated in parentheses. The rates of tolerance induction were scored one month after metamorphosis. —, not thymus grafted.

developed thymuses from stage 48 larvae were not effective (Table 2). Evidently, the tolerance examined in this study is induced in the presence of the developed thymus. Since the similar thymus dependency of tolerance induction (i.e., prevention of autoimmunity) has been reported in mice [14], I believe that there must be some general significance in this phenomenon shared among vertebrate species.

To examine the role of lymphoid cell populations in tolerant animals, the thymus or the spleen of tolerant frogs was grafted to 10 immunodeficient frogs that had been thymectomized at early larval stages. One month after the organ grafting, two adult skin grafts (one JB and one allogeneic) were transplanted simultaneously, and the rejection was observed for 2 months. By grafting the spleen, the tolerant state was transferred along with establishing a normal skin allograft rejecting capacity in all 5 frogs. On the other hand, the tolerant state was never transferred by the thymus graft: thymus grafted 5 frogs rejected not only allografts but also JB grafts within a month. These results indicate that the tolerant state was maintained peripherally by splenic cells but not centrally by thymus cells.

Next, the spleen or the thymus was grafted from tolerant frogs to normal frogs or vice versa, to detect possible suppressing activities of cells and/or other factors in tolerant frogs (Table 3). No change in immunological status was observed in normal frogs after spleen or thymus grafting from tolerant animals, no matter how many organs were used. Likewise, no change was observed when the thymus was grafted from normal to tolerant frogs. On the other hand, the tolerant state was broken in many of the tolerant frogs receiving spleens from normal frogs. The rate of breakdown increased according to the number of spleens grafted. Only 1 out of 10 frogs showed tolerance breakdown by grafting 1 normal spleen. Since it has been confirmed that 1 spleen or a pair of thymuses from a normal frog can restore immune competence in T cell deprived frogs [7, 16], it is reasonable to assume that in tolerant animals some suppressive or regulative activities, though not strong, were exerted on the spleen and the thymus derived from normal animals (Table 3). It can also be considered that the splenic cell populations of tolerant frogs were responsible for at least a part of the suppressive activities, although it was obvious

TABLE 3. Possible suppressive activity in tolerant frogs

grafted organs	recipient	number of animals made or kept tolerant
1 tolerant spleen	normal	0/ 4
2 tolerant spleens	normal	0/ 4
3 tolerant spleens	normal	0/ 4
1 pair tolerant thymuses	normal	0/ 3
2 pairs tolerant thymuses	normal	0/ 3
3 pairs tolerant thymuses	normal	0/ 3
1 normal spleen	tolerant	9/10
2 normal spleens	tolerant	3/10
3 normal spleens	tolerant	0/10
1 pair normal thymuses	tolerant	3/ 3
2 pairs normal thymuses	tolerant	3/ 3
3 pairs normal thymuses	tolerant	3/ 3

About 2 months after metamorphosis, tolerant or normal JJ frogs received either the thymuses or the spleens from normal or tolerant JJ counterparts, respectively. Normal JJ frogs were grafted with adult JB skin at the same time as the organ grafting, while tolerant frogs were not grafted with new one. The fate of the grafted skin in normal animals or that of tolerated skin in tolerant animals were scored 2 months after the organ grafting.

that the spleen cells alone were not strong enough to suppress the graft rejection in normal frogs. Other factors such as antigens themselves and/or humoral regulators may be involved in the maintenance of the tolerance. It should also be addressed in the future study whether the development of these suppressive activities are under the influence of the thymus during metamorphosis.

Despite the present findings, it is apparent that the thymus dependent suppression is not the sole mechanism to make tadpoles tolerant of adult 'self' antigens, because even late thymectomized tadpoles do not usually suffer from autoimmune disease but can normally metamorphose (cf. [2]). Therefore, the present mechanism should be regarded as one of the several fail-safe mechanisms for physiological tolerance induction in metamorphosing tadpoles.

The great advantage in the present system is that the tolerance induction was practically impossible of semixenogeneic JB skin grafts in late thymectomized larvae. This will extremely help us to obtain clear-cut results in experimentation. It is in sharp contrast with the case among allogeneic or semi-allogeneic combinations where tolerance could sometimes be induced even in late thymectomized animals [2]. Moreover, because JB cell has an excellent cellular marker [11] when mixed with JJ cells, the present system will provide an invaluable experimental model in developmental immunology.

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